Cyclosporin effect on noradrenaline release from the sympathetic nervous endings of rat aorta

Paula Tavares, C.A. Fontes Ribeiro, F. Teixeira

Institute of Pharmacology and Experimental Therapeutics, Faculty of Medicine, University of Coimbra, 3049 Coimbra codex, Portugal

Accepted 30 September 2002

Abstract

Arterial hypertension is one of the main side effects of cyclosporin treatment and seems to be due to activation of the sympathetic nervous system. Some authors hypothesized that cyclosporin may act on the sympathetic nervous endings increasing catecholamine release, in agreement with our previous works which demonstrated an increase in rat plasma catecholamine levels after 30 mg/kg per day cyclosporin treatment for 7 weeks. Therefore, the aim of this work was to study the cyclosporin mechanism responsible for that increase in plasma catecholamines. Male Wistar rats were used. Noradrenaline release was performed in vitro experiments after loading rat aorta abdominal segments with 3H-noradrenaline (3H-NA). The release of 3H-NA was measured after electrical stimulation in the presence of 10^{-6} M cyclosporin. In another set of experiments electrical stimulation was replaced by a pulse addition of cyclosporin (10^{-6} M). Another group of rats was treated with 30 mg/kg per day cyclosporin for 7 weeks and catecholamine contents in aorta abdominal segments and adrenals were measured by high performance liquid chromatography system with electrochemical detection (HPLC-ECD). An increase in the 3H-NA release was observed in both types of in vitro experiments. Since cocaine abolished these cyclosporin effects, the obtained results suggest that cyclosporin may act on the catecholamine transporter across the membrane. In addition, after the 7 weeks of cyclosporin treatment, a significant decrease in catecholamine aorta contents was verified but in adrenals there was no difference regarding to controls. However, the dopamine synthesis/degradation ratio measured by the DA/DOPAC ratio suggests an increase in dopamine synthesis. These facts are in agreement with the enhanced plasma catecholamine levels and with the hypothesis of tissue catecholamine depletion. However, they do not explain the increase in plasma adrenaline levels, since adrenaline is a reflex of adrenal activity. The synthesized dopamine in adrenals seems to be unable to reach vesicles and to be metabolized in adrenaline. The observed decrease in HVA adrenal levels may be a consequence of extraneuronal uptake inhibition or inhibition by cyclosporin of the direct α-methylation of DOPAC.

In conclusion, our results suggest that cyclosporin increases catecholamine release from the sympathetic nervous endings by a tyramine-like effect, i.e. by acting directly on the catecholamine transporter of the membrane.

© 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Cyclosporin; Noradrenaline release; Rat aorta; Sympathetic nervous system

1. Introduction

Cyclosporin is an immunosuppressive drug that has considerably improved the survival of transplant patients in recent years. However, several side effects have been associated with cyclosporin treatment, such as hypertension, nephrotoxicity and neurotoxicity. The possible mechanisms underlying cyclosporin-induced hypertension were reviewed by Sandet and Victor [1]. Nevertheless, none of the hypothesis is out of controversy. Thus, the only fact that is in agreement with all of them is that cyclosporin changes vascular reactivity. Concerning noradrenaline and phenylephrine-induced contraction, cyclosporin seems to increase [2-5] or decrease [6-8] vessel contraction. To explain these facts Xue et al. [2] hypothesize that after an inhibition of the α2 adrenoceptors cyclosporin increases contraction by an increase of noradrenaline release from the sympathetic nervous endings. Also Yaris and Tuncer in 1995 [3], indicated cyclosporin as a possible adrenoceptors inhibitor, after the observation that cyclosporin increased noradrenaline levels more than those obtained by electrical stimulation. Moreover, studies on plasma catecholamine levels in human patients and animals treated with cyclosporin have provided controversial results. In this sense, Van den Dopel et al. [9] and Sehested et al. [10] found no changes in plasma noradrenaline levels in patients with kidney transplant and hypertension undergoing treatment.
with cyclosporin. In contrast, also in humans and under similar conditions, Crum et al. [11] observed an increase in plasma adrenaline and noradrenaline levels. Experiments in cyclosporin-treated rats have also afforded conflicting results. In a study following the administration of 5, 10 and 20 mg/kg per day cyclosporin for 3 weeks, Gerkens [12] did not find any changes in plasma catecholamine levels. However, Duruibe et al. [13] described an increase in noradrenaline and adrenaline levels but not of dopamine. More recently, our research group observed a significant increase in rat plasma catecholamine levels (noradrenaline, adrenaline and dopamine) after 7 weeks of treatment with 30 mg/kg per day cyclosporin—Sandimmune Neoral® [14]. As expected, these results were directly correlated with cyclosporin-induced hypertension. The observed effect of cyclosporin on plasma catecholamine levels may be due to alterations in the sympathetic nerve endings of blood vessels, promoted by the immunosuppressive drug. In the light of these findings, the goal of the present work was to study the effect of cyclosporin in the noradrenaline release from the sympathetic nerve endings of blood vessels. We also studied the effect of cyclosporin treatment on the catecholamine contents of rat abdominal aorta and adrenals in order to contribute for a possible explanation of the changes in plasma catecholamine levels and subsequent hypertension.

2. Material and methods

Male Wistar rats (weighing 300–350 g at the beginning of experiments) were housed four to five per cage under a 12 h light/dark cycle in a room with controlled temperature (22 ± 1°C), humidity (50 ± 10%) with food and water available ad libitum. The experimental animals were handled as carefully as possible in order to minimize environmental stress. Animal experiments were carried out in agreement with the European Convention on Animal Care, and the whole research project in which these studies are included received the approval of the Portuguese National Foundation for Science and Technology.

2.1. Experimental procedure

The animals were randomized into two groups: Group I was treated with 30 mg/kg cyclosporin and Group II received only the solvent (control group). Rats were treated daily with cyclosporin for a period of 7 weeks by oral administration. The cyclosporin was the commercial formulation (Sandimmune Neoral®) dissolved in orange juice as indicated for human treatment. Both groups received the same volume of the drug or solvent (0.5 ml).

The cyclosporin dose used in vivo induced a blood concentration similar to that found in humans, as discussed before [15]. Thus, cyclosporin blood levels measured in our treated rats were 1.76 ± 0.06 μM and 0.43 ± 0.11 μM at 14 and 36 h, respectively, after the last administration of 30 mg/kg per day cyclosporin. Moreover, the 10−6 M cyclosporin corresponds approximately to maximal plasma values attained in human pharmacokinetics [16,17]. Based on these results, a 10−6 M cyclosporin concentration was used in all in vitro experiments.

2.2. Dissection and determination of monoamines

After the 7 weeks cyclosporin treatment the animals were sacrificed by pentobarbital sodium overdose. The abdominal segment of aorta as well as adrenals were rapidly removed and cleaned from surrounding tissue. Small segments of abdominal aorta and adrenals were immediately placed into 0.1 M perchloric acid (HClO4) solution and kept overnight. Aliquots of the extract were directly injected in a high performance liquid chromatography system with electrochemical detection (HPLC-ECD).

The chromatographic system consisted of a Gilson Applied Chromatographic System with a 305 model pump and a 231 injection valve model, with a 50 μl loop. A Biopace ODS RP18 analytical column (250 × 4.6, Ø = 5 μm; Bioanalytical Systems Inc., USA) was used and separation was made possible by using an isocratic solvent system consisting of an acetate–citrate buffer (sodium acetate 0.1 M, citric acid 0.1 M), containing sodium octane sulfonate (0.5 mM), Na2EDTA (0.15 mM), dibutylamine (1 mM) and 10% methanol. A flow rate of 1 ml/min was maintained and detection of the chromatographed catecholamines achieved by using a 141 Gilson electrochemical detector model (650 nV).

2.3. Release of 3H-noradrenaline

2.3.1. Incubation with 3H-noradrenaline

The strips of abdominal aorta were immersed in a Krebs physiologic solution of the following composition (which was also used for superfusion): mmol/l: NaCl 118.67, KCl 5.36, CaCl2 1.90, NaH2PO4 0.67, NaHCO3 11.1, glucose 6.027 and ascorbic acid 0.057 mmol/l were added to the solution which was maintained at 37°C and bubbled with 95% O2 and 5% CO2. Tissues were first exposed for 30 min to pargyline (1 mM) to inhibit monoamine oxidase (MAO), and then incubated with 3H-noradrenaline (3H-NA) (for 1 h in small beakers containing 5 ml medium with (→)3H-NA (0.23 μmol/l).

2.3.2. Spontaneously and electrically-induced release of 3H-noradrenaline

After incubation, the tissues were transferred into 1 ml glass chambers and perfused with 3H-NA-free medium from bottom to top at a rate of 0.8 ml/min. Two protocols were performed.

2.3.2.1. Protocol 1: electrically-induced 3H-noradrenaline release.

After a washout period (of 75 min), the perfusion
The vascular segments were heat (100 °C in a dry bath) with 200 μl H₂O₂ (30 vol.) plus 200 μl HClO₄, 30% during 15 min. To the solubilized samples scintillation fluid was added, and the radioactivity measured in a Packard 2000 spectrometer provided with dpm correction.

In both protocols using [3H]NA a HPLC-ECD control was performed in order to determine that in the final of the experiments we, in fact, measured noradrenaline and not other substances like noradrenaline metabolites.

2.5. Preparation of cyclosporin

The majority of authors used ethanol to dissolve cyclosporin. However, this solvent could have some undesired effects. To avoid these pitfalls, we used dimethyl sulfoxide (DMSO) as solvent. In all control experiments DMSO was also added. The possible morphological-induced alterations of this cyclosporin solution were tested in smooth muscle cell cultures. DMSO (in the percentage used) showed no effects on cell morphology or viability (data not showed).

2.6. Chemicals

Cyclosporin and Sandimmune Neoral® were supplied by Novartis Farma, Portugal. [3H]-NA (specific activity = 12 Ci/mmol) was obtained from Amersham (USA). The scintillation fluid (UNIVERSOL) was obtained from ICN (Costa Mesa). All the other chemicals were obtained from Sigma, St. Louis, MO, USA.

2.7. Statistics

Fisher’s protected least significant difference (PLSD) test preceded by one-way ANOVA were applied. Probability levels of less than 0.05 were regarded as statistically significant.

3. Results

In a previous published work we demonstrated that an increase in plasma catecholamine levels was observed after cyclosporin administration [14]. In those animals the increase in plasma catecholamine levels was correlated with an increase in blood pressure. Moreover, cyclosporin induced arterial hypertension after the first week of treatment. Since we hypothesize that the increase in plasma catecholamine levels was due to a cyclosporin action on the vascular sympathetic nervous endings, the aorta catecholamine contents were measured in the present work. After 7 weeks of 30 mg/kg per day cyclosporin treatment, a significant decrease in catecholamine contents (noradrenaline, adrenaline and dopamine) was observed in abdominal aorta segments (Fig. 1). To further understand the cyclosporin effect on...
Fig. 1. Catecholamine contents in rat abdominal aorta segments after 7 weeks of treatment with solvent (control) (n = 10) or with CsA 30 mg/kg per day (n = 12). Catecholamines (noradrenaline (NA), adrenaline (AD) or dopamine (DA)) were determined by HPLC-ECD. Data shown are means ± S.E.M. ∗P < 0.05 related to control.

Table 1
Electrically-induced release of [3H]-noradrenaline from rat aorta segments

<table>
<thead>
<tr>
<th>Release fraction ratio</th>
<th>Control</th>
<th>Control + cocaine</th>
<th>Cyclosporin</th>
<th>Cyclosporin + cocaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3/S2</td>
<td>0.847 ± 0.042</td>
<td>0.974 ± 0.087</td>
<td>1.109 ± 0.093*</td>
<td>0.875 ± 0.007</td>
</tr>
<tr>
<td>S4/S2</td>
<td>0.802 ± 0.031</td>
<td>0.852 ± 0.073</td>
<td>1.081 ± 0.143*</td>
<td>0.817 ± 0.049</td>
</tr>
<tr>
<td>S5/S2</td>
<td>0.656 ± 0.220</td>
<td>0.894 ± 0.109</td>
<td>0.795 ± 0.084</td>
<td>0.766 ± 0.038*</td>
</tr>
<tr>
<td>Aorta (dpm/mg tissue)</td>
<td>4305.6 ± 157.9</td>
<td>3402.5 ± 165.8*</td>
<td>2725.0 ± 189.0*</td>
<td>3913.1 ± 364.7</td>
</tr>
</tbody>
</table>

Cyclosporin and/or cocaine were added before S3 and remained for all the experiment. In each period of stimulation the release fraction was calculated according to the expression indicated in Section 2. Values represent mean ± S.E.M. of five independent experiments performed in triplicate.

∗P < 0.05 related to control.
#P < 0.05 related to cyclosporin.

vascular noradrenaline release, [3H]-NA loading experiments were performed. Thus, [3H]-NA was released by electrical stimulation, in the presence of cyclosporin, or by cyclosporin itself. Both [3H]-NA release protocols showed that cyclosporin increases [3H]-NA release. After electrical stimulation, cyclosporin increased both S3/S2 and S4/S2 release fraction ratios, without changes of the S5/S2 ratio (Table 1). When cocaine was added to the organ bath, the calculated fraction ratios became similar to controls. Cocaine alone, as expected, show a slight increase in [3H]-NA release when compared to control (Table 1). In addition, aorta [3H]-NA contents, after the electrically-induced efflux, were decreased, in agreement with the described release results (Table 1). When the electrical stimulation was replaced by the direct cyclosporin addition to the organ bath [3H]-NA release also increased (Fig. 2). Once more, cocaine abolished this cyclosporin effect. These results, from both protocols, may indicate a cyclosporin tyramine-like effect. In fact, when cyclosporin was replaced by tyramine results showed

Fig. 2. Drug-induced release of [3H]-noradrenaline from rat abdominal aorta segments. Cyclosporin and/or cocaine were added in E1 and E2, except in control (only with the solvent) and experiments with tyramine. In each period of stimulation the percent of [3H]-noradrenaline released was calculated according to the expression indicated in Section 2. Values represent mean ± S.E.M. of five independent experiments performed in triplicate. *P < 0.05 related to control of five experiments. #P < 0.05 related to cyclosporin.
Fig. 3. Abdominal aorta contents of 3H-norepinephrine (percent of 3H-norepinephrine not released) after the experiments indicated in Fig. 2. Values were calculated according to the expression indicated in Section 2. Values represent mean ± S.E.M. of five independent experiments performed in triplicate. ∗P < 0.05 related to control of five experiments.

Moreover, the accumulated percent of 3H-NA in aorta segments reinforces this behavior (Fig. 3). All these results seem to suggest that cyclosporin increases 3H-NA release from the sympathetic nervous endings.

The 3H-NA in vitro studies could explain the increase in noradrenaline plasma levels and the decrease in vascular noradrenaline contents of cyclosporin-treated rats. However, the increased adrenaline plasma levels may only be explained by cyclosporin-induced changes in adrenals. When the adrenal catecholamine contents were measured, from cyclosporin-treated animals, no significant changes were observed when compared to controls (Fig. 4). However, 3,4-dihydroxyphenylacetic acid (DOPAC) values significantly increased whereas homovanillic acid (HVA) values decreased (Fig. 5). The sum DOPAC + DA (indicator of synthesis) as well as the DOPAC/DA ratio (indicator of metabolism) showed a significant increase when compared to control (Table 2).

Table 2
<table>
<thead>
<tr>
<th>Indicators of dopamine (DA) synthesis and degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
</tr>
<tr>
<td>DOPAC/DA (×100)</td>
</tr>
<tr>
<td>DOPAC + DA (ng/mg)</td>
</tr>
</tbody>
</table>

Synthesis was evaluated by the sum of DOPAC and DA; degradation was evaluated by the DOPAC/DA ratio. Values represent mean ± S.E.M. ∗P < 0.05 related to control.
4. Discussion

Several mechanisms, including the activation of the sympathetic nervous system, have been proposed to explain the cyclosporin-induced hypertension. However, like other explanations this hypothesis is also not free of controversy. For instance, Stein et al. [18] showed in humans an impaired vasodilation rather than sympathetic activation or enhanced vasconstriction, which may be an important mechanism for the alterations of the vascular tone that occurs after long-term cyclosporin administration. Nevertheless, behind all the controversy that surrounds cyclosporin-induced hypertension there is one common point: a change in vascular reactivity in response to several agents, namely noradrenaline. However, the way of this change is also contradictory in the literature. In response to noradrenaline or phenylephrine cyclosporin seems to increase [2–5] or decrease [6–8] vascular contraction. Several possible mechanisms have been proposed for the increased sympathetic nerve activity: direct release of the transmitter induced by cyclosporin from sympathetic nerves [2] or potentiation of responses to nerve stimulation [19,20]. According to the hypothesis proposed by Xue et al. [2], our results also showed an increase in the noradrenaline release. Since aorta segments are poorly enervated, the experiments were performed with exogenous noradrenaline (3H-NA). In our experiments, after electrical stimulation the presence of cyclosporin increased 3H-NA release and decreased 3H-NA contents in the vascular segments. It seems that cyclosporin may increase the noradrenaline release by acting through the membrane noradrenaline transporter. To test this hypothesis, the 3H-NA studies were performed in the presence of cocaine. The results showed that in the presence of cocaine cyclosporin was unable to induce any increase on 3H-NA release. As expected, cocaine by itself increases 3H-NA release. Thus, these results seem to suggest that cyclosporin may have tyramine-like effect. When the experiments were repeated without electrical stimulation but with addition of cyclosporin we verified the same behavior, i.e. an increase in 3H-NA release. Once more this was similar to the tyramine effect. So, as it happens with tyramine or amphetamines, cyclosporin may also use the uptake 1 to enter into the axoplasm. The hypothesis that cyclosporin could also enter by passive diffusion through the membrane (like tyramine in high concentrations) is not probable since cocaine abolishes the cyclosporin effect. Once in the cytoplasm, cyclosporin enters axoplasmic vesicles and like tyramine or amphetamines decreases the vesicle pH gradient [21]. This will decrease 3H-NA protonation and increase 3H-NA release to the cytoplasm and rapidly from this space to outside. Therefore, cyclosporin seems to act more upon the noradrenaline transporter than in the plasma membrane, which is in agreement with contraction studies in response to potassium where cyclosporin has no effect (unpublished data from the authors). On the contrary, observations of Lamb and Webb [19], in rat tail arteries, indicate that 8.3 × 10^{-6} M cyclosporin appears to be acting at the level of the plasma membrane potential. The decrease in the membrane potential increases both release of noradrenaline from adrenergic nerve ending and smooth muscle responses to applied noradrenaline. In our study, electrical stimulation increases nervous endings stimulation with a consequent enhancement of noradrenaline transport activity, as well as the probable cyclosporin tyramine-like effect. In addition, cyclosporin has no effect on potassium-induced contraction (data not shown).

In the light of our results, after repeated cyclosporin treatment vessels were expected to be depleted of catecholamines. Few works have addressed the effect of cyclosporin on catecholamine tissue contents. In 1995, Pestana et al. [22] reported an accumulation of DA in rat kidney tissue after 14 days of treatment with 50 mg/kg per day cyclosporin. In the present work, after 7 weeks of cyclosporin treatment a decrease of the abdominal aorta catecholamine contents were observed. These data are in agreement with our hypothesis that cyclosporin may have a tyramine-like effect. This effect could also partially justify the increase in plasma catecholamines previously reported [14]. The increase in plasma adrenaline found before [14] is difficult to explain with this hypothesis, since plasma adrenaline levels reflect the amounts of adrenaline synthesized and released by adrenal glands. We found no significant changes in adrenaline contents in the adrenals of cyclosporin-treated animals as compared with the controls. The same results in adrenals were found as regards the other catecholamines assayed (noradrenaline and dopamine). Despite this, on analyzing the dopamine synthesis/degradation ratio, as measured by dopamine metabolite levels, and the relations DOPAC/DA and DOPAC/DA+HVA, some differences were observed. The results obtained in the group of rats treated with 30 mg/kg per day cyclosporin suggest an increase in dopamine synthesis. For some reason, however, this dopamine (or at least not all the dopamine synthesized) was unable to enter neuronal vesicles to be metabolized into noradrenaline and adrenaline. Some of it was converted into DOPAC outside the vesicles leading to the verified increase in DOPAC levels. These findings are in agreement with the results obtained by Pestana et al. [22] in rat kidney tissue after 50 mg/kg per day cyclosporin treatment. According to our results, it would appear that vesicles have some difficulty in incorporating dopamine. A possible reason could be the hypothesis discussed above that cyclosporin may have a direct effect on catecholamine transport across membranes. Moreover, in the adrenals of this group of rats a decrease in HVA levels was also observed, suggesting that this concentration of cyclosporin might also interfere in the conversion of DOPAC to HVA, inhibiting extraneuronal uptake and subsequent metabolization by COMT (catechol-o-methyltransferase), or directly inhibiting DOPAC-o-methylation.

In spite of the absence of differences in adrenals catecholamine contents we could not exclude the fact that cyclosporin could increase both adrenal catecholamine...
vasoconstriction. This could be also be an explanation for our in vivo results. However, we also demonstrated that more than this effect on the activation of sympathetic nerve activity, cyclosporin has also a direct effect over the sympathetic nervous endings.

In conclusion, our results suggest that cyclosporin increase the noradrenaline release from sympathetic nervous endings, probably by interfering on the catecholamine transport across membrane rather than by an action on the plasma membrane potential. This fact may explain the increase in catecholamine plasma levels and the consequent arterial hypertension, as well as the increase in adrenal DOPAC.

Acknowledgements

We are grateful to Novartis Inc. for their kind collaboration in this project. This work was supported by a grant from the Portuguese National Foundation for Science and Technology (Praxis Program)—PRAXIS/PSAU/C/SAU/57/96.

References